Fibrinogen up-regulates the expression of monocyte chemoattractant protein 1 in human saphenous vein endothelial cells

Suzanne L. HARLEY and Janet T. POWELL¹

Department of Vascular Surgery, Imperial College School of Medicine at Charing Cross, Charing Cross Hospital, Fulham Palace Road, London W6 8RF, U.K.

High concentrations of fibrinogen in plasma have been associated with an increased risk of saphenous vein graft pathology. We have investigated the ability of fibrinogen to up-regulate the expression of monocyte chemoattractant protein 1 (MCP-1) in cultured human saphenous vein endothelial cells (HSVEC) isolated from saphenous vein. Increasing concentrations of fibrinogen (0–4 μ M) stimulated a 20-fold increase in MCP-1 secretion within 4 h. Incubation of HSVEC with 2 μ M fibrinogen for 4 h also caused a 2-fold increase in the MCP-1-to-glyceraldehyde-3-phosphate dehydrogenase mRNA ratio. The fibrinogen-mediated MCP-1 secretion fell to basal levels after preincubation of HSVEC with the complex of fibrinogen fragments D and E but remained unchanged after preincubation of

HSVEC with either fibrinogen fragment E, s-ICAM-1 or the pentapeptide GRGDV. In contrast, fibrinogen fragment D acted as a potent inhibitor of fibrinogen-mediated MCP-1 secretion. Labelled fibrinogen fragment D bound to HSVEC with a K_d of 6.5 μ M. These findings indicate that fibrinogen, at physiological concentrations, uses an epitope on the fibrinogen D domain to bind to a receptor on HSVEC to up-regulate MCP-1 expression and secretion. This receptor seems to be distinct from intercellular adhesion molecule 1 and the integrins previously recognized as fibrinogen receptors.

Key words: endothelium, fragment D, ICAM-1, vein bypass.

INTRODUCTION

Increased plasma fibrinogen concentrations are associated with the development of stenotic lesions in saphenous vein bypass grafts [1–3]. The ability of fibrinogen to elicit vasomotor effects in saphenous vein rings [4] heightens the possibility that fibrinogen interacts directly with the cells of the vein graft wall to facilitate the development of the intimal hyperplasia, which underlies vein graft stenosis. In these stenotic lesions there is abnormal intimal migration and proliferation of vascular smooth-muscle cells, deposition of extracellular connective tissue and macrophage infiltration [5,6].

Chemokines, a superfamily of small (6–15 kDa) inducible, proinflammatory cytokines, act primarily as chemoattractants and activators of specific types of leukocyte [7–9]. Monocyte chemoattractant protein 1 (MCP-1), a strong chemoattractant for monocytes [10], accounts for most of the monocyte chemoattractant activity secreted by endothelial cells and vascular smooth-muscle cells *in vitro* [11]. Resting endothelial cells constitutively express very low levels of MCP-1 [12]. The interaction of monocytes with endothelial cells cultured from human umbilical vein (HUVEC) has been shown to increase endothelial expression of both MCP-1 and interleukin 8 [12]. A range of cytokines, including interleukin 1 β (IL-1 β) and tumor necrosis factor α (TNF- α), also up-regulate the expression of MCP-1 [11,13,14].

Fibrinogen is a 340 kDa plasma protein with a normal circulating concentration of 4–6 μ M. It is a glycoprotein consisting of two sets of three polypeptide chains (A α , B β and γ) joined close to the N-terminal end by a knot of disulphide bonds. A complex of the two main fibrinogen fragments D and E (fg.D–fg.E complex) is produced from fibrinogen after digestion with plasmin, which removes two small peptide chains [fibrino-

peptides A and B (FpA and FpB)] from the N- and C-termini of the A α chains [15]. The individual fragments, two fg.D fragments, from the C-terminal end of the coiled-coil region, and a single fg.E fragment, containing the disulphide knot, can be further isolated from this complex.

Previous studies have indicated that fibrinogen acts as a ‘bridging molecule’ to promote the adhesion of THP-1 cells to HUVEC [16]. Fibrinogen increased THP-1 adhesion to HUVEC by bridging Mac-1 on the THP-1 cells to intercellular adhesion molecule 1 (ICAM-1) on IL-1 β -activated HUVEC [16]. The peptide γ_3 , consisting of a sequence near the C-terminus of the γ -chain (N¹¹⁷NQKIVNLKEKVAQLEA¹³³), inhibited fibrinogen binding to ICAM-1 on HUVEC by 40–50% [17]; this peptide lies within fg.D. This role of fibrinogen as a bridging ligand was observed only at subphysiological concentrations (less than 1 μ M); at higher concentrations fibrinogen did not increase THP-1 cell adhesion to HUVEC [16].

The presence of macrophages in vein graft lesions, together with the association between high concentrations of plasma fibrinogen and vein graft stenosis, led us to investigate the hypothesis that the binding of fibrinogen to ICAM-1 on saphenous vein endothelium stimulates the secretion of MCP-1. Experiments reported here, using endothelial cells cultured from saphenous vein (HSVEC), support part of this hypothesis.

EXPERIMENTAL

Materials

Tissue culture media were obtained from Life Technologies (Paisley, Renfrewshire, Scotland, U.K.). Endothelial growth supplement, antibiotics, fibrinopeptides A and B, mRNA micro isolation kit and the mouse anti-caldesmon monoclonal antibody were obtained from Sigma (Poole, Dorset, U.K.). Heparin

Abbreviations used: ALENA, ALENAESQNLVKKIQKN; DIG, digoxigenin; fg.D, fibrinogen fragment D; fg.D–fg.E, complex of fibrinogen fragments D and E; fg.E, fibrinogen fragment E; FpA, fibrinopeptide A; FpB, fibrinopeptide B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSVEC, human saphenous vein endothelial cells; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; IL-1 β , interleukin 1 β ; MCP-1, monocyte chemoattractant protein 1; TNF- α , tumour necrosis factor α ; γ_3 , N¹¹⁷NQKIVNLKEKVAQLEA¹³³.

¹ To whom correspondence should be addressed (e-mail j.powell@ic.ac.uk).

Monoparin was from CP Pharmaceuticals (Wrexham, Clwyd, U.K.). Fibronectin was a gift from Dr. Jan van Mourik (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). Plasminogen and streptokinase were kindly supplied by Professor David Lane (Department of Haematology, Imperial College School of Medicine at Charing Cross, London, U.K.). Fibrinogen was obtained from Kabi-Pharmacia or from Enzyme Research Laboratories (Swansea, W. Glamorgan, U.K.). Mouse anti-(human MCP-1), mouse anti-(human TNF- α) neutralizing antibody, Fab and F(ab)₂ fragments of mouse anti-(ICAM-1) monoclonal antibody (clone 6.5B5; IgG₁ κ), soluble ICAM-1 and the MCP-1 gene probe cocktail were obtained from R&D Systems (Abingdon, Oxon, U.K.). Mouse anti-(human ICAM-1) monoclonal antibody (clone 6.5B5; IgG₁ κ), antibodies against von Willebrand factor were from Dako (High Wycombe, Bucks., U.K.). Antibodies against CD31 were from Serotec (Oxford, U.K.). The γ_3 peptide, residues 117–133 of the fibrinogen γ chain, the scrambled γ_3 peptide ALENAEVQNVLVKKIQKN (ALENA) [17] and the pentapeptide GRGDV were synthesized in house (Advanced Biotechnology Centre at Charing Cross). The IL-1 β ELISA kit was obtained from AMS Biotechnology (Europe) Ltd. (Whitney, Oxon, U.K.). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene probe was obtained from Calbiochem Novabiochem (Nottingham, U.K.). Streptavidin-alkaline phosphatase conjugate was obtained from Vector Laboratories (Peterborough, Cambs., U.K.), the dioxigenin (DIG) oligonucleotide labelling kits and luminescence detection kits were from Boehringer Mannheim (Lewes, East Sussex, U.K.) and X-ray film from Genetic Research (Braintree, Essex, U.K.) and Texas-Red labelling kit and Ribo Green from Molecular Probes (via Cambridge Bioscience, Cambridge, U.K.).

Human saphenous vein endothelial cells (HSVEC)

HSVEC were isolated from human saphenous vein by digestion with collagenase and were cultured as described previously on fibronectin-coated plastic [18]. The use of saphenous vein was approved by the Riverside Research Ethics Committee. HSVEC were cultured in M199 culture medium supplemented with 10% (v/v) heat-inactivated human serum, 17 i.u./ml heparin monoparin, 15 μ g/ml endothelial cell growth supplement, 50 i.u./ml penicillin and 50 μ g/ml streptomycin. No fibrinogen was detected by immunonephelometry in the human serum. Cells were used at passage 2 or 3 and transferred into serum-free medium for all experiments investigating the effect on MCP-1 secretion of fibrinogen, fibrinogen fragments, antibodies against ICAM-1 or other reagents.

Human MCP-1 ELISA

A 96-well flat-bottomed plate (Dynex, Ashford, Middx, U.K.) was coated with mouse anti-(human MCP-1) monoclonal antibody (2 μ g/ml), diluted in carbonate coating buffer [25 mM Na₂CO₃/75 mM NaHCO₃ (pH 9.6)] by incubation for 16 h at 4 °C. All wells were washed three times with wash buffer [PBS/0.5% (v/v) Tween 20], blotted dry and incubated for 1 h at 37 °C with PBS containing 10% (v/v) foetal calf serum. The wells were rinsed three times with wash buffer and incubated with samples or standards (recombinant human MCP-1, 0–10 ng/ml) for 2 h at 37 °C. After three rinses with wash buffer, goat anti-(human MCP-1) antibody (2 μ g/ml), diluted in wash buffer, was added and the plate was incubated at 37 °C for 1 h. The plate was rinsed three times with wash buffer and rabbit anti-(goat Ig) biotinylated antibody (0.2 μ g/ml) was added for 1 h at 37 °C. The plate was washed three times and 100 μ l of streptavidin-

alkaline phosphatase (1:1000) was added to each well and incubated for 30 min at 37 °C. The plate was washed three times and *p*-nitrophenyl phosphate substrate solution added (100 μ l per well). The yellow colour was left to develop for 60 min at 37 °C before A_{405} was measured with a Labsystems Uniskan II ELISA reader. The coefficient of variation for the assay was 10% in the concentration range 0–5 ng/ml.

Isolation of HSVEC mRNA

HSVEC were incubated with fibrinogen (0–4 μ M) or IL-1 β (5 ng/ml) for 4 h, washed in PBS and mRNA was isolated with the mRNA micro isolation kit. In brief, cells in a 75 cm² flask were washed with PBS before the addition of lysis buffer (1 ml). The lysates were agitated at 45 °C for 20 min before the addition of 5 M NaCl (63 μ l) and aspiration of the mix through a 21-gauge needle. The mRNA was isolated by chromatography on oligo(dT)-cellulose spin columns. After elution the mRNA was precipitated by sodium acetate/ethanol in the presence of glycogen carrier. The yield of mRNA (8–25 μ g) was quantified with RiboGreen.

Slot-blotting

Two pieces of heavy absorbent paper presoaked in 20 $\times$ SSC [3 M NaCl/0.3 M sodium citrate (pH 7)] were placed on top of the slot-blot vacuum unit. One sheet of positively charged nylon membrane was placed underneath the slot-blot sample wells. mRNA from HSVEC was diluted 1:4 to a final concentration of 50% (v/v) formamide and 6.7% (v/v) formaldehyde in SSC, incubated for 15 min at 68 °C and cooled on ice. Each well of the membrane was washed with 1 ml of 10 $\times$ SSC before loading the mRNA (diluted with 2 vol. of 20 $\times$ SSC). Then each well was rinsed twice with 10 $\times$ SSC (1 ml) and suction was continued for 5 min to dry the membrane. The membrane was removed, air-dried and baked for 2 h at 80 °C. Oligonucleotide probes were labelled with digoxigenin-dideoxy-UTP with a DIG oligonucleotide 3' end-labelling kit. Membranes were hybridized overnight at 54 °C with the required DIG-labelled oligonucleotide (DIG-MCP-1 probe cocktail; 25 ng/ml) or DIG-GAPDH (12.5 pmol). The membrane was developed with a DIG luminescence detection kit for nucleic acids. Medical RX X-ray film was exposed to the membrane for 15 min before development. Quantification of mRNA was performed by densitometry with a Chromscan 3 (Joyce-Loebl, Gateshead, Tyne and Wear, U.K.).

Preparation of fibrinogen fragments

Fibrinogen (Kabi-Pharmacia) was digested with plasmin and fg.D; the fg.D–fg.E complex was purified as described previously [9]. In brief, fibrinogen (20 mg/ml) was digested overnight with plasminogen (0.143 mg/ml) and streptokinase (286 units/ml) in the presence of 10 mM CaCl₂. Aprotinin (50 i.u./ml) was added to inhibit proteolysis, before gel filtration on a column of Sephacryl S200, developed with 0.05 M Tris/HCl, pH 7.4, containing 2 mM CaCl₂ and 10 i.u./ml aprotinin. A broad peak, eluting in the range 250–120 kDa, was collected and concentrated through a membrane with a 30 kDa exclusion limit. The high-molecular-mass pool was termed the fgD.fgE complex [9]. fg.D was purified from this pool by FPLC on a Mono Q column (a strong anion-exchanger). The Mono Q column was developed with CaCl₂ gradient (0–1.4 M): fg.D was eluted at 0.3 M CaCl₂. This method yielded only low amounts of fg.E, for which an alternative preparative method was therefore used. Fibrinogen (20 mg/ml) was dissolved in double-distilled water and digested

for 48 h with plasminogen (0.143 mg/ml) and streptokinase (285.7 units/ml) in the presence of CaCl_2 (10 mM) at room temperature. Aprotinin (50 i.u./ml) was added and the digest was incubated for a further 30 min at 60 °C; the insoluble material was removed by ultracentrifugation at 98 500 *g* for 1 h at 10 °C. The supernatant, containing fg.E, was removed and further purified by FPLC. The purity of fg.D and fg.E was checked by SDS/PAGE.

Binding of labelled fragment D to HSVEC

fg.D was labelled with Texas Red through a C6 spacer arm in accordance with the manufacturer's instructions (Molecular Probes, Eugene, OR, U.S.A.). From the A_{595} the degree of labelling was calculated as 5–6 mol/mol fg.D. HSVEC were grown to confluence in 96-well plates and washed three times with ice-cold serum-free medium. The cells were incubated with labelled fg.D (0–10 μM) in medium 199 containing 0.2% BSA, heparin (17 i.u./ml) and endothelial cell growth supplement (15 $\mu\text{g}/\text{ml}$) at 4 °C for periods from 0 to 3 h. At the end of the incubation, the medium was aspirated and the HSVEC were rapidly washed three times with ice-cold PBS containing 0.2% BSA and 1 mM CaCl_2 . The fluorescence was quantified with the Cytofluor (Perkin Elmer, Warrington, Cheshire, U.K.).

RESULTS

Fibrinogen stimulates the secretion of MCP-1 from HSVEC

Incubation of HSVEC with fibrinogen (0–4 μM) for 6 h resulted in a concentration-dependent increase in MCP-1 secretion, with maximum effect being achieved at 2 μM fibrinogen (Figure 1). This effect was seen in HSVEC isolated from 17 separate saphenous vein donors. Fibrinogen (4 μM) significantly increased MCP-1 secretion from a basal level of 0.07 ± 0.02 ng/ml to 0.20 ± 0.09 ng/ml at 2 h, 1.8 ± 0.6 ng/ml at 4 h and 2.5 ± 1.3 ng/ml at 6 h (means $\pm$ S.E.M., $n = 5$). In comparison, incubation of HSVEC with IL-1 β (5 ng/ml) for 4 h increased MCP-1 secretion to 3.8 ± 1.2 ng/ml. MCP-1 secretion was similar when fibrinogen (0–4 μM) was incubated with HSVEC in the presence or absence of hirudin (1 i.u./ml), an inhibitor of thrombin activity (Figure 1). There was no discernable degradation of fibrinogen α chain (as determined by SDS/PAGE) or fibrinopeptide release (as determined by capillary electrophoresis) after incubation with HSVEC for 4 h in the presence of hirudin. All further investigations were performed with an incubation period of 4 h, so that significant concentrations of MCP-1 were secreted, and in the presence of hirudin as a precaution against exogenous thrombin activity. When HSVEC were incubated with fibrinogen (4 μM) in the presence of cycloheximide (10 μM) or actinomycin D (2 $\mu\text{g}/\text{ml}$) the release of MCP-1 decreased from 3.8 ± 0.5 to 0.2 ± 0.02 ng/ml ($n = 4$) and 0.7 ± 0.1 ng/ml ($n = 4$) respectively, (analysis of variance $P < 0.02$).

Fibrinogen does not stimulate MCP-1 secretion from HSVEC via IL-1 β or TNF- α

IL-1 β concentrations were measured in the conditioned medium to investigate whether fibrinogen was acting indirectly via this cytokine. Incubation of HSVEC with fibrinogen did not cause the secretion of IL-1 β , which was trivial under basal conditions and at all fibrinogen concentrations (0.02–0.03 ng/ml, $n = 3$). Antibody neutralization studies were performed to evaluate whether TNF- α was a mediator in the fibrinogen-dependent release of MCP-1. When HSVEC were incubated with TNF- α (5 ng/ml) for 4 h, substantial amounts of MCP-1 (4.4 ± 0.4 ng/ml) were released. In the presence of TNF- α and a neutralizing

Figure 1 Fibrinogen increases MCP-1 secretion from HSVEC

HSVEC were incubated with fibrinogen (0.3–4 μM) for 6 h in the presence (black bars) or absence (hatched bars) of hirudin (1 i.u./ml). The results are means $\pm$ S.E.M., with cells from 17 donors. Both in the presence and absence of hirudin there is a significant concentration-dependent increase in MCP-1 secretion (analysis of variance with Bonferroni's correction, $P < 0.001$).

Figure 2 Fibrinogen increases MCP-1 mRNA levels in HSVEC

HSVEC were incubated with fibrinogen (0.3–4 μM) or IL-1 β (5 ng/ml) for 4 h before mRNA isolation. MCP-1 (A) and GAPDH (B) mRNA were identified by hybridization with DIG-labelled oligonucleotides.

mouse anti-(human TNF- α) monoclonal antibody (5 ng/ml) the amount of MCP-1 released was decreased from 4.4 ± 0.4 to 1.2 ± 0.9 ng/ml ($n = 3$). In contrast, no decrease was seen in the fibrinogen-mediated MCP-1 release in the presence or absence of the mouse anti-(human TNF- α) monoclonal antibody (5 ng/ml): MCP-1 release was 2.9 ± 0.37 and 3.8 ± 1.3 ng/ml respectively ($n = 3$). These results indicate that fibrinogen is not acting indirectly, through either IL-1 β or TNF- α , to stimulate the release of MCP-1 from HSVEC.

Fibrinogen increases MCP-1 mRNA levels in HSVEC

Small amounts of MCP-1 mRNA were isolated from HSVEC under basal conditions; however, after incubation of HSVEC with fibrinogen (0.3–4 μM) for 4 h, increased amounts of MCP-1 mRNA were observed (Figure 2A). IL-1 β (5 ng/ml) was used as a positive control. The ratio of MCP-1 to GAPDH

Table 1 Effect of fibrinogen, its fragments and peptides on MCP-1 secretion from HSVEC

Results are means $\pm$ S.E.M.; the number of different cell donors (n) is shown. Significant increase in MCP-1 secretion, * $P = 0.002$; ** $P = 0.03$ (Wilcoxon signed rank tests).

HSVEC incubated for 4 h with	n	MCP-1 secretion (ng/ml)
Medium only	11	0.1 ± 0.02
+ Fibrinogen ($4 \mu\text{M}$)	11	$2.5 \pm 0.4^*$
+ fg.D–fg.E ($4 \mu\text{M}$)	4	0.1 ± 0.02
+ fg.D ($0.1 \mu\text{M}$)	5	0.6 ± 0.1
+ fg.D ($4 \mu\text{M}$)	11	$0.6 \pm 0.2^{**}$
+ fg.E ($4 \mu\text{M}$)	11	0.06 ± 0.01
+ FpA ($3 \mu\text{M}$)	11	0.08 ± 0.02
+ FpB ($3 \mu\text{M}$)	9	0.05 ± 0.04
+ γ_3 ($25 \mu\text{M}$)	13	0.07 ± 0.02
+ GRGDV	6	0.08 ± 0.03

Table 2 Effect of fibrinogen on MCP-1 secretion after preincubation of HSVEC with fibrinogen fragments, peptides and other proteins

The F(ab)₂ was from a monoclonal antibody against ICAM-1. Results are means $\pm$ S.E.M.; the number of different cell donors (n) is shown. Significant decrease in MCP-1 secretion, * $P < 0.01$, ** $P = 0.04$.

Preincubation of HSVEC for 30 min with	n	MCP-1 secretion after incubation with fibrinogen for 4 h (ng/ml)
Medium		2.5 ± 0.4
+ fg.D ($4 \mu\text{M}$)	6	$0.4 \pm 0.2^*$
+ fg.E ($4 \mu\text{M}$)	4	2.6 ± 0.3
+ fg.D–fg.E ($4 \mu\text{M}$)	4	$0.06 \pm 0.01^*$
+ γ_3 ($25 \mu\text{M}$)	7	$1.3 \pm 0.7^{**}$
+ ALENA ($25 \mu\text{M}$)	5	1.7 ± 0.5
+ soluble ICAM-1 ($100 \mu\text{g/ml}$)	3	2.1 ± 0.6
+ F(ab) ₂ ($25 \mu\text{g/ml}$)	3	1.9 ± 0.7

(Figures 2A and 2B) mRNA measured by scanning densitometry was 1.53 under basal conditions, increasing to 4.88 after incubation of HSVEC with IL-1 β (5 ng/ml) for 4 h. The MCP-1-to-GAPDH mRNA density ratios at 0.3, 1, 2 and 4 μM fibrinogen were 2.28, 2.24, 3.81 and 2.96 respectively ($n = 2$). MCP-1 mRNA seemed to have increased by a maximum of 2-fold in the presence of fibrinogen, compared with a 3-fold increase in the presence of IL-1 β .

Fibrinogen binds via its D-domain on HSVEC to mediate MSP-1 release

FpA and FpB ($3 \mu\text{M}$), the peptide γ_3 ($25 \mu\text{M}$), the pentapeptide GRGDV ($40 \mu\text{M}$) and fg.E ($4 \mu\text{M}$) did not stimulate MCP-1 secretion; fg.D at low concentration ($0.1 \mu\text{M}$) increased MCP-1 secretion from HSVEC by a modest 3–4 fold, from 0.15 ± 0.04 to 0.6 ± 0.1 ng/ml ($n = 5$) but higher concentrations of fg.D (up to $4 \mu\text{M}$) did not have greater effect (Table 1). The MCP-1 secretion stimulated by low concentrations ($0.1 \mu\text{M}$) of fg.D was potently inhibited by preincubation of HSVEC with γ_3 ($25 \mu\text{M}$), whereas ALENA ($25 \mu\text{M}$) had no effect; MCP-1 secretion was 0.1 ± 0.1 and 0.5 ± 0.2 ng/ml respectively ($n = 5$). Preincubation of HSVEC with either fg.D ($4 \mu\text{M}$) or the fg.D–Fg.E complex ($4 \mu\text{M}$) potently inhibited the fibrinogen-mediated release of MCP-1 (Table 2); fg.E and GRGDV were

A **B** **Figure 3** Binding of Texas Red-labelled fg.D to HSVEC

(A) Saturable binding; (B) data transformed into a Scatchard plot. Binding studies were performed at 4 °C and each well contained 18 000–20 000 endothelial cells. The data shown are the average of quadruplicate measurements at each experimental point. The apparent dissociation constant (K_d) was calculated to be $6.5 \mu\text{M}$. Abbreviation: AU, arbitrary units.

Table 3 Fibrinogen-mediated secretion of MCP-1 is attenuated after culture of HSVEC in the presence of $4 \mu\text{M}$ fibrinogen

Results are means $\pm$ S.E.M. ($n = 4$).

Previous exposure of HSVEC to fibrinogen	MCP-1 secretion (ng/ml) after HSVEC culture for 4 h in	
	Medium alone	Medium + fibrinogen
HSVEC cultured in fibrinogen-free medium for 16 h	0.07 ± 0.01	2.5 ± 0.3
HSVEC cultured with fibrinogen for 16 h	0.5 ± 0.1	0.7 ± 0.2
HSVEC cultured with fibrinogen for 16 h, then in fibrinogen-free medium for 4 h	0.4 ± 0.1	1.2 ± 0.4
HSVEC cultured with fibrinogen for 16 h then in fibrinogen-free medium for 24 h	$0.12 \pm 0.05^*$	2.3 ± 0.8

* Return to basal MCP-1 secretion; under all other conditions MCP-1 secretion is increased.

ineffective. Preincubation of HSVEC with γ_3 attenuated the fibrinogen-mediated release of MCP-1, although to a smaller extent than fg.D (Table 2); the scrambled γ_3 peptide, ALENA (25 μ M), had less effect. These experiments suggested that fibrinogen (at physiological concentrations), via an epitope on the D domain, binds to a receptor on HSVEC. The binding of fg.D to bovine pulmonary artery endothelial cells has been reported previously [19]. The binding of Texas Red-labelled fg.D (4 μ M) to confluent HSVEC at 4 °C increased slowly to give maximum binding after 3 h, without morphological perturbation of the endothelial monolayer. Binding seemed to be saturable at very high concentrations of labelled fg.D (Figure 3A). Labelled fg.D could be displaced by either excess unlabelled fg.D (20 μ M) or partly displaced by 4 μ M fibrinogen (results not shown). A Scatchard plot of bound or free labelled fg.D against bound fg.D (Figure 3B) was linear, indicating only one important binding site in the concentration range studied. The K_d at 4 °C was calculated as 6.5 μ M.

Cross-linking of ICAM-1 causes limited MCP-1 secretion from HSVEC

Incubation of HSVEC with a monoclonal antibody against ICAM-1 (35 μ g/ml) stimulated only low levels of MCP-1 secretion (0.6 ± 0.4 ng/ml, $n = 6$). An IgG-subtype-matched control antibody, mouse anti-caldesmon, had no effect. The F(ab)₂ fragment (25 μ g/ml) of the same ICAM-1 antibody also stimulated MCP-1 secretion to a similar extent to the parent antibody (0.5 ± 0.1 ng/ml) but the Fab fragment (25 μ g/ml) did not. Preincubation of HSVEC with the fg.D–fg.E complex (4 μ M) did not diminish MCP-1 secretion in response to incubation with antibodies against ICAM-1. Conversely, preincubation of HSVEC with either monomeric soluble ICAM-1 (100 μ g/ml) or the F(ab)₂ fragment (25 μ g/ml) did not attenuate significantly the fibrinogen-mediated release of MCP-1 (Table 2).

Pre-exposure of HSVEC to fibrinogen decreases subsequent fibrinogen-mediated MCP-1 secretion

To investigate the effect on MCP-1 release of long-term exposure of HSVEC to fibrinogen, HSVEC were cultured in serum-free medium with added fibrinogen (4 μ M) for 16 h (in the presence of hirudin). After 16 h the cells were washed thoroughly and fibrinogen-free medium [containing 5% (v/v) human serum to maintain cell viability] was added. The cells were maintained in this medium for 'rest' periods of 0–24 h, before being washed and then incubated further with fibrinogen (4 μ M, 4 h) in serum-free medium. After 'rest' periods of 0–6 h, there was only a modest increase in MCP-1 secretion after HSVEC were rechallenged with fibrinogen (Table 3). After a 'rest' period of 24 h, HSVEC rechallenged with fibrinogen again responded with a very marked increase in secretion of MCP-1 (Table 3). These results indicate that fibrinogen receptors on HSVEC, which signal for increases in MCP-1 secretion, might be down-regulated or desensitized when cells are cultured continuously in the presence of fibrinogen.

DISCUSSION

Fibrinogen induced, in a concentration-dependent manner, the secretion of MCP-1 from cultured HSVEC. This increased secretion of MCP-1 (from 0.1 ng/ml in the absence of fibrinogen to approx. 3 ng/ml at 4 μ M fibrinogen) was not mediated by the release of either IL-1 α or TNF- β from HSVEC. Incubation of HSVEC with fibrinogen was also associated with a 2-fold increase

in MCP-1 mRNA. Actinomycin D inhibited the fibrinogen-mediated MCP-1 secretion only partly, suggesting that both increased levels of mRNA and increased rates of transcription are necessary to achieve the 30-fold increases in MCP-1 secretion. Preincubation of HSVEC with fibrinogen fragment complex fg.D–fg.E completely inhibited the fibrinogen-mediated MCP-1 secretion. These findings confirmed the first part of our hypothesis that fibrinogen, binding to a specific receptor on HSVEC, stimulated the secretion of MCP-1.

Many studies have looked at the acute effects of fibrinogen on endothelial and circulating blood cells. There are few reports of the chronic effects of fibrinogen on endothelial cells, which are usually cultured in the presence of serum but in the absence of fibrinogen. We observed that after HSVEC had been cultured in the presence of 4 μ M fibrinogen (physiological concentration) the response to fibrinogen was attenuated. In particular, when HSVEC were cultured in the absence or presence of 4 μ M fibrinogen for 24 h before the 4 h incubation with fibrinogen, the secretion of MCP-1 was diminished from 2.5 ± 0.3 ng/ml to 0.7 ± 0.2 ng/ml. The response to fibrinogen could be recovered if cells were returned to a fibrinogen-free medium for 24 h. Therefore the specific fibrinogen receptor on HSVEC signalling for MCP-1 secretion seems to become down-regulated by chronic exposure to fibrinogen. This fibrinogen receptor might become reactivated by sudden increases in plasma fibrinogen, as observed during the acute-phase response.

The second part of our hypothesis, that the important fibrinogen receptor on HSVEC, involved in stimulating MCP-1 secretion, was ICAM-1 was not proved. Fibrinogen at very low concentrations binds to the first domain of ICAM-1 at a site distinct from the binding site for lymphocyte-function-associated-antigen-1-binding site, which also is located on the first domain of ICAM-1 [20]. The peptide γ_3 , from the fibrinogen D domain, inhibits the binding of fibrinogen to ICAM-1 [16,17]. All our observations are consistent with the hypothesis that an epitope in the fg.D domain binds to a receptor on HSVEC to stimulate MCP-1 secretion. The high MCP-1 secretion observed at physiological concentrations of fibrinogen (4 μ M) was potentially inhibited by plasmin digestion products of fibrinogen containing fg.D. This is consistent with our observation that at high concentrations fg.D binds to a single site on HSVEC with a K_d of 6.5 μ M. Treatment of HSVEC with either a cross-linking antibody against the binding site for lymphocyte-function-associated antigen 1 on ICAM-1 or low concentrations of fg.D did cause a limited increase in MCP-1 secretion. However, the amount of MCP-1 secreted was less than one-quarter of that observed when HSVEC were incubated with fibrinogen. Interestingly, this ability of fg.D to act as a weak agonist could be inhibited by γ_3 . This could account for the weak inhibition of fibrinogen-mediated MCP-1 secretion by this peptide. These results imply that at low concentrations of fibrinogen, its interaction with ICAM-1 weakly stimulates MCP-1 secretion. The experimental conditions used for the fg.D binding experiments would not have permitted evaluation of any high-affinity binding site for fibrinogen on HSVEC that was manifest at low concentrations of fibrinogen. At the higher, physiological concentrations of fibrinogen, the principal fibrinogen receptor on HSVEC, involved in MCP-1 secretion, does not seem to be ICAM-1.

Several effects of fg.D on endothelial cells, including the redistribution of actin in stress fibres, increased permeability and monolayer disorganization and the activation of fibrinolysis, have been described previously [19,21,22]. These effects on cellular morphology were manifest only after many hours and not within the 4 h period that we used. The first of these studies

demonstrated that binding of fg.D to endothelial cells, although a K_d was not calculated. We observed the specific and saturable binding of labelled fg.D to HSVEC, with a K_d at 4 °C of 6.5 μ M. This is similar to the reported K_d (1.5 μ M) for the binding of fibrinogen to monolayers of HUVEC [23] and the fibrinogen concentration that maximally increases MCP-1 secretion from HSVEC.

We have been unsuccessful in our attempts to characterize the receptor on HSVEC to which fibrinogen binds at physiological concentrations. However, we have presented evidence to show that this receptor is unlikely to be ICAM-1 or an integrin, although both $\alpha_v\beta_3$ and $\alpha_v\beta_1$ are expressed in cultured endothelial cells. fg.D, which is both a weak agonist to stimulate MCP-1 secretion and a potent inhibitor of the fibrinogen-mediated secretion of MCP-1, does not contain the sequence RGD, although the sequence KQAGDV, which binds the platelet integrin $\alpha_{IIb}\beta_3$, is present at the C-terminus of the γ chain. Moreover, the peptide GRGDV failed to inhibit fibrinogen-mediated MCP-1 secretion from HSVEC. The promiscuous nature of fibrinogen in binding to a diverse variety of receptors, including integrins, ICAM-1, calreticulin and VE-cadherin [24–27], underscores the difficulty of identifying the principal receptor on HSVEC that triggers MCP-1 secretion.

When saphenous vein is used as an arterial bypass conduit, the venous endothelium becomes exposed to new haemodynamic stresses, including increased shear stress and cyclic radial, circumferential and longitudinal deformations [28]. These new haemodynamic stresses might stimulate MCP-1 gene expression and alter the concentration of adhesion molecules on the endothelium [29,30]. ICAM-1 is one of the adhesion molecules that are rapidly up-regulated on saphenous vein endothelium exposed to arterial flow conditions [31]. Because the interaction of fg.D with ICAM-1 seems to cause modest MCP-1 secretion, in the newly implanted vein graft the binding of fibrinogen degradation products to ICAM-1 to stimulate MCP-1 secretion could assume increased importance. After surgery there will be a rise in circulating fibrinogen concentration with the acute-phase response, which might act synergistically to promote MCP-1 secretion and facilitate monocyte recruitment into the vein graft wall. Such secretion of MCP-1 could be necessary for the proper maturation of the vein graft [32] or could assume pathological significance.

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